AD_____

Award Number: DAMD17-97-1-7239

TITLE: A Cell Culture Model for Understanding Estrogen Receptor Regulation in Normal and Malignant Cells

PRINCIPAL INVESTIGATOR: Virginia Novaro, Ph.D.
Dr. Mina Bissell

CONTRACTING ORGANIZATION: University of California
Berkeley, California 94720

REPORT DATE: October 2001

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2001	Annual (30 Sep 00 - 29 Sep 01)

4. TITLE AND SUBTITLE
A Cell Culture Model for Understanding Estrogen Receptor Regulation in Normal and Malignant Cells

5. FUNDING NUMBERS
DAMD17-97-1-7239

6. AUTHOR(S)
Virginia Novaro, Ph.D.
Dr. Mina Bissell

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California
Berkeley, California 94720

E-Mail: vnovaro@lbl.gov

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

One of the major obstacles to study the contribution of estrogen receptor (ER) to the initiation and progression of breast cancer is the lack of an appropriate culture model that duplicates the estrogenic effects seen in the normal gland in vivo. We postulate that the correct expression and function of ER depends on an appropriate microenvironment that preserves cell-cell and cell-matrix interactions. Here we determined that the extracellular matrix (ECM) modulates the expression and function of the ER. The stimulation of the ER-mediated response by the presence of cell-matrix interactions involves the α6 integrin subunit. Using quantitative RT-PCR and western blots we also demonstrated that both normal and tumorigenic mouse mammary cell lines in culture increased ERα expression levels in response to ECM and that those levels are higher in the tumorigenic cell line. Moreover, the presence of estradiol decreased the expression of its own receptor in both cell types, two-fold in normal cells and fifteen-fold in tumorigenic cells.

Therefore, we believe that the regulatory effect of ECM is exerted, at least in part, by modulating both the ER-mediated response and/or ERα levels itself. In addition, we have developed an in vitro culture system for mouse mammary cells isolated from the gland, which partly prevents the loss of ERα expression normally seen in standard culture conditions. This improved technique relies on culturing the cells in 3D cultures inside collagen I gels or on top of Matrigel instead of 2D monolayers, and the addition of lactogenic hormones.

These results suggest that the approach that we have chosen to analyze the regulation of ER function by a three-dimensional matrix is promising. The regulation of ER by ECM could be a crucial step in the progression of breast cancer from an ER-positive to an ER-negative tumor, which leads to a hormone-independent and more aggressive cancer phenotype. In addition, manipulating ER expression in mammary cells by controlling the extracellular environment might be a powerful tool to understand the mechanisms of estrogen dependent tumor formation and it can contribute to the development of new therapies that target the points of contact between the cancer cell and its microenvironment.

14. SUBJECT TERMS
Breast Cancer

15. NUMBER OF PAGES
6

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Cover 1

SF 298 2

Table of Contents 3

Progress Report 4

Comments 6

References 6

Appendices ..... n/a

Addendum to the Annual Progress Report presented on October 2000
Covers Progress in Year 2 (October 2000 only)

"A Cell Culture Model for Understanding Estrogen Receptor Regulation in Normal and Malignant Cells" (award number DAMD17-97-1-7239).

Postdoctoral Fellow: Virginia Novaro

In order to study the effect of the extracellular matrix on the estrogen receptor regulation, we started using a mouse mammary epithelial cell line "Scp2ERETKCAT" (described in the previous report) in order to look for an ER-mediated response to ECM. The Scp2ERE-TK-CAT cell line derives from Scp2 cells (a cell line already established in our laboratory (1)) and it is a cell line stably transfected with an ER transcriptional reporter construct containing an estrogen response element (ERE) from the xenopus vitellogenin A2 gene, upstream of the CAT gene (2). As shown in the previous report, when those cells were cultured on top of EHS matrix (reconstituted basement membrane derived from Englebreth-Holm-Swarm (EHS) tumor), they adopt an acini-like structure, whereas on plastic they adopt a typical flattened morphology. When the cells were growing on top of EHS, the ER-mediated response (CAT reporter activity) increases, even in the absence of estradiol, (+ ethanol), Figure 1 (as in previous report). When estradiol is added to the medium, the reporter activity is increased on plastic and on top of EHS. The addition of an antagonist of ER, ICI-182,780 blocked the effect of estradiol but not the effect of matrix, indicating that part of the response to matrix is estradiol independent.

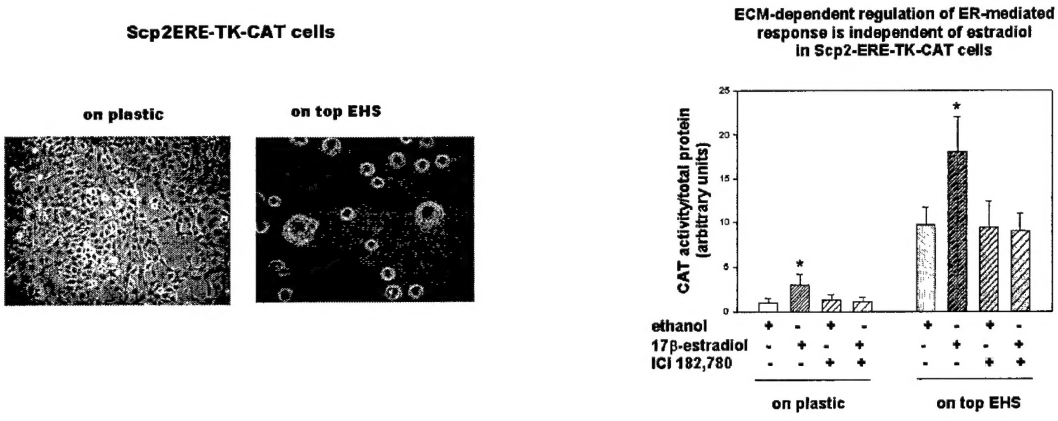

*Figure 1 Top.Photomicrographs showing the morphological cell change in Scp2-ERE-TK-CAT cells cultured for 4 days on plastic or on top of EHS (matrigel) in DMEM/F12 medium. The cells on plastic form a flattened monolayer, whereas the cells on top of EHS aggregate and form acini-like structures. Bottom: In the conditions mentioned above, I tested the reporter activity (CAT assay using Stratagene kit) in the presence of ethanol (control), 17β-estradiol 10^{-8}M, or the antagonist ICI 182,780 10^{-7}M. After 4 days in culture the cells were lysed and the proteins extracted to perform the CAT assay following manufacturer instructions. Replicates=4. *:p<0.01 17β-estradiol vs. ethanol.*

Progress report:

Our last set of experiments towards reach our goal in understanding the mechanisms by what ECM regulates ER expression and function, were the identification of one of the integrin receptors involved in this regulatory effect of matrix. We proved that the estrogen-dependent ER response to matrix involves the α6 integrin subunit (Figure 2), since an antibody against the α6 integrin subunit blocked the increase on the reporter activity in the presence of estradiol when the cells were cultured on top of EHS, without affecting cell shape. α6 integrin subunit has previously shown at our lab to be involved in β-casein synthesis but not in cell shape change induced by laminin in Scp2 cells (3).

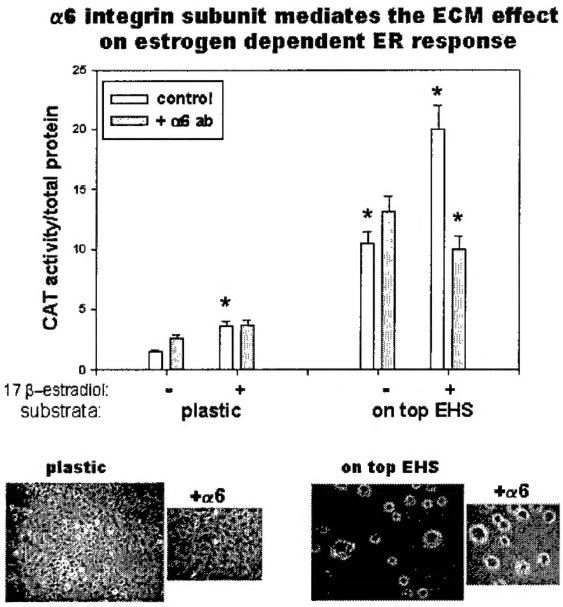

Figure 2. α6 integrin subunit mediates the ECM effect on estrogen-dependent ER response.
*Scp2-ERE-TK-CAT cells were grown on plastic or on top of EHS for 4 days in the absence or presence of 10 μg/ml of α6 integrin blocking antibody (PharMingen). The reporter activity is affected by the presence of the α6 integrin antibody only when the cells were grown on top of EHS and in the presence of estradiol. Bottom: Cell morphology is not altered by the antibody. Replicates=3. *:p<0.05 + estradiol vs. – estradiol (plastic); EHS on top vs. plastic (+/-estradiol); +α6 ab vs. control (on top EHS + estradiol).*

My previous results (presented in the previous report) and new results presented here on Table 1 signal that both mRNA and protein levels of ERα are regulated by ECM, indicating that ECM regulates ER-mediated response, not just by activating the ER, but also by increasing the amount of ER expression, acting at the transcriptional level of the ERα gene.

Table 1. ERα mRNA levels in Scp2 cells growing on different substrata. *:p<0.05 vs. plastic

Substrata	Plastic	drip EHS	on top EHS
ERα/G3PDH	0.55+/-0.12	0.83+/-0.15 *	1.2+/-0.19 *

***Table 1.** EHS regulates ERα levels at the transcriptional level.*
Quantification of ERα mRNA levels in Scp2 cells grown on plastic, drip EHS or on top EHS by quantitative PCR (Light Cycler) using G3PDH as an internal standard.

Table 2. ERα protein levels in Scp2 cells growing on different substrata *p:<0.05 vs. plastic

Substrata	plastic	drip EHS	on top EHS
ERα/E-cadherin	1.02 +/- 0.05	2.8 +/- 0.9 *	3.2 +/- 1.02

***Table 2.** ERα protein level is up-regulated in the presence of matrix.*
*Quantification of western blots performed with protein lysates from Scp2 cells cultured for 4 days on different substrata. To detect ERα, we used H-184 polyclonal ab (Santa Cruz) and to detect E-cadherin we used a monoclonal ab (Transduction laboratories). E-cadherin was used as a loading control in each experiment. Replicates n=5. *:p<0.05 vs. plastic.*

Comments:

I started with this award from the Army Medical Research and Materiel Command on March 1999. With the present results and the results reported previously (October 2000) I complete the report of the studies developed during the period when I was the PI in this fellowship, March 1999 – October 2000.

I have determined that the expression and function of ERα is modulated partially by changes in cell shape, from flattened to rounded form, but fundamentally by cell-ECM interactions. Also that the presence of cell-matrix interactions stimulates ER-mediated response in mouse mammary epithelial cells in two ways, one independent of estradiol and another one estradiol-dependent, this last one through the α6 integrin subunit.

In addition, I would like to emphasize that I have established a protocol to isolate mouse mammary epithelial cells from the gland and culture them in conditions that partly prevent the loss ER-expression. These conditions include ECM components (Matrigel) and lactogenic hormones (hydrocortisone and prolactin) (Figure 8 previous report). This discover is of crucial importance to our ongoing research. I consider it essential to extrapolate the results obtained using cell lines to the *in vivo* situation. The use of primary cultures is a step in that direction, since the behavior of primary cultures should be more representative of the mammary gland physiology than established cell lines.

Our new results presented here, indicate that the α6 integrin blocking antibody, the integrin that recognize laminin in the ECM (3) is not sufficient to completely block the effect of EHS, suggesting that other ECM components (like collagens) should be involved in the up-regulation of ER-mediated response.

References:

1. Desprez,PY. Roskelley C, Campisi J., Bissell M. Isolation of functional cell lines from a mouse mammary epithelial cell strain: The importance of basement membrane and cell-cell interaction. *Mol.& Cel. Differentiation.* **1(1)**, 99-110 (1993).

2. Klein-Hitpass,L., Schorpp,M., Wagner,U. & Ryffel,G.U. An estrogen-responsive element derived from the 5' flanking region of the Xenopus vitellogenin A2 gene functions in transfected human cells. *Cell* **46**, 1053-1061 (1986).
3. Muschler J., Lochter A., Roskelley CD, Yurchenco P and Bissell MJ. Division of labor among the α6β4 integrins and an E3 laminin receptor to signal morphogenesis and β-casein expression in mammary epithelial cells. *Molecular Biology of the Cell.* **10**, 2817-2828 (1999).